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Heterogeneity of the SR-dependent Inward Na^+-Ca^{2+} Exchange Current in the Heavily Ca^{2+}-buffered Rat Ventricular Myocytes

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Voltage-sensitive release mechanism was pharmacologically dissected from the Ca^{2+}-induced Ca^{2+} release in the SR Ca^{2+} release in the rat ventricular myocytes patch-clamped in a whole-cell mode. SR Ca^{2+} release process was monitored by using forward-mode Na^+-Ca^{2+} exchange after restriction of the interactions between Ca^{2+} from SR and Na^+-Ca^{2+} exchange within micro-domains with heavy cytosolic Ca^{2+} buffering with 10 mM BAPTA. During stimulation every 10 s with a pulse roughly mimicking action potential, the initial outward current gradually turned into a huge inward current of -12.9±0.5 pA/pF. From the inward current, two different inward I_{NCX}s were identified. One was 10 μM ryanodine-sensitive, constituting 14.2±2.3%. It was completely blocked by $CdCl_2$ (0.1 mM and 0.5 mM) and by Na^+-depletion. The other was identified by 5 mM $NiCl_2$ after suppression of I_{CaL} and ryanodine receptor, constituting 14.8±1.6%. This latter was blocked by either 10 mM caffeine-induced SR Ca^{2+}-depletion or 1 mM tetracaine. IV-relationships illustrated that the latter was activated until the peak in 30∼35 mV lower voltages than the former. Overall, it was concluded that the SR Ca^{2+} release process in the rat ventricular myocytes is mediated by the voltage-sensitive release mechanism in addition to the Ca^{2+}-induced-Ca^{2+} release.

Key Words: Voltage-sensitive release mechanism, Ca^{2+}-induced Ca^{2+} release, Heavy Ca^{2+}-buffering, Na^+-Ca^{2+} exchange, SR, Rat heart

INTRODUCTION

Myocardial contraction is induced by an increase in the intracellular Ca^{2+} (Ca^{2+}_i) level, which couples the electrical excitation with the mechanical contraction (excitation-contraction coupling, ECC). As the sarcoplasmic reticulum (SR) provides 70∼92% of the Ca^{2+}_i rise depending on the species (Bers et al, 1996), the mechanism mediating SR Ca^{2+} release is considered as a key to the ECC.

In cardiac myocytes, it has long been known that SR Ca^{2+} release is triggered by the external Ca^{2+} entering the cell through the voltage-gated L-type Ca^{2+} channels during the action potential, which is known as the 'Ca^{2+}-induced Ca^{2+} release (CICR)' (Fabiato, 1983; Lopez-Lopez et al, 1995). Ca^{2+} entered through other routes such as the T-type Ca^{2+} channel (Sipido et al, 1998; Zhou & January, 1998), the reverse-mode Na^+-Ca^{2+} exchange (NCX) (Leblanc & Hume, 1990; Sipido et al, 1997) and possibly Na^+ channels (Santana et al, 1998; Piacentino et al, 2000) is also able to induce CICR although its physiological contribution appears to be minor (Sipido et al, 1998; Zhou & January, 1998).

Another possibility has recently been raised in triggering the SR Ca^{2+} release. Using a two-step protocol in guinea-pig ventricular myocyte shortening, Ferrier and Howlett (1995) reported the external Ca^{2+} influx-independent fraction of the contraction in addition to the L-type Ca^{2+} current (I_{CaL})-dependent contraction. The I_{CaL}-dependent contraction elicited a bell-shaped contraction-voltage relationship in the voltage range equivalent to that of the I_{CaL} starting from -40 mV to +80 mV with a peak around 0 mV. In contrast, the external Ca^{2+} influx-independent contraction was activated in more negative voltage ranges than the I_{CaL}-dependent contraction, beginning from -70~-65 mV with a peak around -20 mV. After a peak, however, the contraction remained constant eliciting a sigmoid voltage- contraction relationship (Ferrier & Howlett, 1995; Ferrier et al, 1998; Howlett et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Ferrier & Howlett, 2001). This external Ca^{2+} influx-independent contraction was completely blocked by tetracaine, although very low concentration of ryanodine elicited a partial blocking (Ferrier & Howlett, 1995; Ferrier et al, 1998; Howlett et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Ferrier & Howlett, 2001). Therefore, the voltage- sensitive release mechanism (VSRM) has been proposed as a possible mechanism mediating the SR Ca^{2+} release in this external Ca^{2+} influx-independent but stimulation voltage-dependent con-

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ABBREVIATIONS: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; NCX, Na+-Ca^{2+} exchange; I_{NCX}, Na^+-Ca^{2+} exchange current; I_{CaL}, L-type calcium current.; CICR, calcium-induced calcium release.

traction (Ferrier & Howlett, 1995; Ferrier et al, 1998). However, the VSRM is very fastidious, so that it requires not only physiological conditions such as physiological temperature of 37°C and no internal dialysis, but also cAMP-dependent phosphorylation for its activation (Ferrier & Howlett, 1995; Ferrier et al, 1998; Howlett et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Ferrier & Howlett, 2001).

All the reports supporting the putative VSRM so far came from one group of researchers and one other group in cooperation with them. In contrast, all the reports by other groups seriously questioned actual presence of the putative VSRM in cardiac myocyte, although they were not many (Piacentino et al, 2000; Griffiths & MacLeod, 2003; Trafford & Eisner, 2003). The major uncertainty raised so far is concerned the fact that they cannot completely rule out the external Ca^{2+} influx in cardiac myocyte even in the presence of very high concentrations of Ca^{2+} channel blockers. It has actually been shown that the application of even 0.5 mM $CdCl_2$ or 60μM nifedipine cannot completely block the I_{CaL} (Griffiths & MacLeod, 2003; Trafford & Eisner, 2003). Furthermore, this problem would be more serious after cAMP-dependent phosphorylation (Piacentino et al, 2000). It is also expected that the external Ca^{2+} is transported into the cell through reverse-mode NCX at more positive potentials. Based on these considerations, therefore, they claimed that the putative VSRM would actually be a result of the CICR induced by these external Ca^{2+}s (Piacentino et al, 2000; Griffiths & MacLeod, 2003; Trafford & Eisner, 2003).

The putative VSRM has another serious flaw: The actual Ca^{2+} release process from the SR has not yet been identified in the case of the putative VSRM, which is crucial to prove its presence (Sipido, 2003). All the experiments dealing with the putative VSRM monitored ventricular myocyte shortening and/or global $Ca^{2+}{}_i$ transient only, regardless of whether they are in support on the putative VSRM or not. The actual Ca^{2+} release process from the SR has never yet been monitored in the putative VSRM research.

Whatever the mechanism is involved, the $Ca^{2+}{}_i$ raised is extruded from cell via forward-mode NCX in addition to its transport into the SR again by Ca^{2+}-ATPase in order to relax the heart. During extrusion of Ca^{2+} from cell, NCX generates an inward current against Ca^{2+} movement according to its stoichiometry of 3 Na^+ against 1 Ca^{2+} (Reeves & Hale, 1984). Therefore, $Ca^{2+}{}_i$ could be monitored by measuring this inward I_{NCX} in the heart (Weber et al, 2002). Furthermore, if it were possible that the interactions between the NCX and Ca^{2+} could be restricted within close vicinity to the SR Ca^{2+} release channels (ryanodine receptor, RyR), this inward I_{NCX} would then represent a SR Ca^{2+} release process in the heart. On the basis of this idea in this study, the cytosolic Ca^{2+} was heavily buffered with 10 mM BAPTA after patch-clamp in a whole cell-configuration in the enzymatically isolated single rat ventricular myocytes. In this situation, global $Ca^{2+}{}_i$ transient disappeared completely during stimulation with a 135 ms-ramp pulse every 10 s from +50 mV to a holding potential of -85 mV in the rat ventricular myocytes. Therefore, it is expected that the heavy cytosolic Ca^{2+} buffering with 10 mM BAPTA limited the Ca^{2+} diffusion time strongly enough, so that the inward I_{NCX}, if any, would denote an extrusion of the Ca^{2+} from cell right after its release from SR before its diffusion to the global $Ca^{2+}{}_i$. In this situation, SR Ca^{2+}-dependent inward I_{NCX} was pharmacologically dissected into two fractions according to its external Ca^{2+} influx-dependence. Therefore, this study was undertaken to clarify the presence of the putative VSRM by the directly monitored SR Ca^{2+} release process in the heart.

The results showed that the SR Ca^{2+}-dependent inward I_{NCX} was pharmacologically separable into two fractions: the external Ca^{2+} influx-dependent fraction and the external Ca^{2+} influx-independent fraction. One was sensitive to ryanodine, while the other was not sensitive to ryanodine but to tetracaine. The I-V relationship showed that the latter was activated until the peak in 30～35 mV lower voltage range than the former that was activated in an equivalent range to the I_{CaL} activation. Therefore, it was concluded that the SR Ca^{2+} release process in the rat ventricular myocytes monitored by using the forward-mode Na^+-Ca^{2+} exchange is mediated by the voltage-sensitive release mechanism in addition to the Ca^{2+}-induced-Ca^{2+} release.

METHODS

Cell isolation

Ventricular myocytes from Sprague- Dawley rats of either sex, weighing about 250 g, were isolated according to the method described by Mitra and Morad (1985). Animal welfare was in accordance with the institutional guidelines of the Yonsei University. Briefly, rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, ip). Hearts were quickly excised and perfused at 6 mL/min in a Langendorff apparatus, first with Ca^{2+}-free Tyrode solution composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$ and 10 glucose, pH 7.4 at 37°C for 8 min, then with 25 ml of Ca^{2+}-free Tyrode solution containing 35 mg of collagenase (type A, Boehringer) and 3 mg of protease (type XIV, Sigma) for 15 min, and finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 8 min. The ventricle of the digested heart was then cut into several sections and subjected to gentle agitation to dissociate cells. The freshly dissociated cells were stored at room temperature in a Tyrode solution containing 0.2 mM $CaCl_2$ and were used within 10 h of isolation. The compositions of the Tyrode solution (in mM) were 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$ and 10 glucose, pH=7.4 at 37°C. All the experiments were performed at room temperature.

Current recording

The rat ventricular myocytes were patch-clamped in a whole-cell configuration and held at -85 mV with a superfusion of the Tyrode solution containing 2 mM Ca^{2+} throughout the experiment. The myocytes were depolarized every 10 s by a 135 ms-ramp pulse from +50 mV to a holding potential of -85 mV (at -1 mV/ms). The current generated by the test pulse was measured using an Axopatch 200B amplifier (Axon Instruments, CA, USA). The generation of the voltage-clamp protocols and data acquisition were carried out using pCLAMP software (version 8, Axon Instruments, CA, USA). The resistance of the patch electrodes was 2.0～3.0 MΩ when filled with an internal solution composed of (in mM) 10 NaCl, 105 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 10 HEPES, 5 Mg-ATP, 1 $CaCl_2$, 0.1 cAMP, and 10 mM BAPTA (titrated

to pH=7.2 with CsOH). KCl was replaced with Cs and TEA in order to block the K^+ currents. cAMP was added to prevent SR Ca^{2+} depletion. The membrane capacitance was measured using pCLAMP software (version 8, Axon Instruments, CA, USA). The inward current obtained during the test pulse was integrated (Area Under the Curve, AUC) to calculate the charge influx and expressed into the charge influx through the unit membrane (pC/pF) and its % value to the control charge influx through the unit membrane obtained after application of vehicle only.

$Ca^{2+}{}_i$ measurement

Isolated rat ventricular myocytes were loaded with fura-2/AM (5μM) for 60 min and placed in a chamber on the stage of a fluorescence microscope (Olympus, Tokyo, Japan). During stimulation with the test pulse, fluorescence was measured in single ventricular myocytes using a dual-wavelength fluorescence photomultiplier system (the Ratio Fluorescence system, Photon Technology International Inc., Lawrenceville, NJ, USA) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Qualitative changes in $[Ca^{2+}]_i$ were inferred from the ratio of the fluorescence intensity at the two wavelengths.

Drug application

The drugs were diluted from stock solutions to the required concentrations in a 2 mM $CaCl_2$-added Tyrode solution, where KCl was omitted and 0.1 mM DIDS was added to suppress the K^+ and Ca^{2+}-activated Cl^- currents, respectively. In the case of Tyrode solution with Ca^{2+}-free (0Ca), Na^+-free (0Na) or Na^+- and Ca^{2+}-free (0Na-0Ca), $CaCl_2$ was replaced with equimolar EGTA, and NaCl was replaced with equimolar LiCl in the above solution. The drugs were applied for 10 s until the end of the test pulse, using a rapid drug exchanger (time required for exchange $<$100 ms).

RESULTS

In this experiment, it was intended to trace the changes in the SR Ca^{2+} release process by using NCX as a Ca^{2+} sensor in the isolated single rat ventricular myocytes. In order to make NCX to interact only with the Ca^{2+}s immediately after its release from SR, the Ca^{2+} diffusion time was limited as short as possible by heavy buffering of the cytosolic Ca^{2+} with high BAPTA added in the pipette after a patch-clamp in whole-cell configuration. The test pulse, a ramp pulse from +50 mV to -85 mV for 135 ms roughly mimicking an action potential, was applied every 10 s to activate the SR Ca^{2+} release in the myocytes held at -85 mV.

Fig. 1A shows the changes in the global $Ca^{2+}{}_i$ transient after cytosolic Ca^{2+}-buffering with variable concentrations of BAPTA. As expected, the magnitude of global $Ca^{2+}{}_i$ transient decreased in a BAPTA concentration-dependent manner and completely disappeared at 10 mM. After all, 10 mM BAPTA limited the Ca^{2+} diffusion time short enough, so that the Ca^{2+} released from SR during stimulation could not reach the global cytosol, indicating that the Ca^{2+}-related response, if any, would be restricted within very narrow micro-domains separated from the global $Ca^{2+}{}_i$ in the presence of 10 mM BAPTA. Therefore, 10 mM BAPTA was used in the following experiments.

The current was also dramatically changed during stimulation with the test pulse after heavy Ca^{2+}-buffering with 10 mM BAPTA. Immediately after the patch, an outward current was elicited in the rat ventricular myocytes. However, as shown in Fig. 1B, as the test pulse was repeated every 10 s, the outward current gradually turned into an inward current. This change required 8～12 minutes to equilibrate. The maximum inward current after an equilibration was -12.9±0.5 pA/pF (n=53). Two factors could be considered to cause this change. One is the gradual suppression of the K^+ outward currents caused by CsCl+ TEA replacing KCl in the pipette. The other is the gradual enhancement of I_{CaL} caused by the BAPTA-induced gradual loss of the Ca^{2+}-induced L-type Ca^{2+} channel inhibition (Adachi-Akahane et al, 1996; Adachi-Akahane et al, 1997; Sham, 1997) and also by the cAMP added in the pipette.

In order to clarify the SR Ca^{2+} release during the test pulse, 10μM ryanodine was applied to 10 myocytes. As shown in Fig. 2A, the vehicle, KCl-omitted and 0.1 mM DIDS-added Tyrode solution, completely suppressed the holding current and mildly enhanced the inward current during the test pulse, transmitting 1.068±0.042 pC/pF.

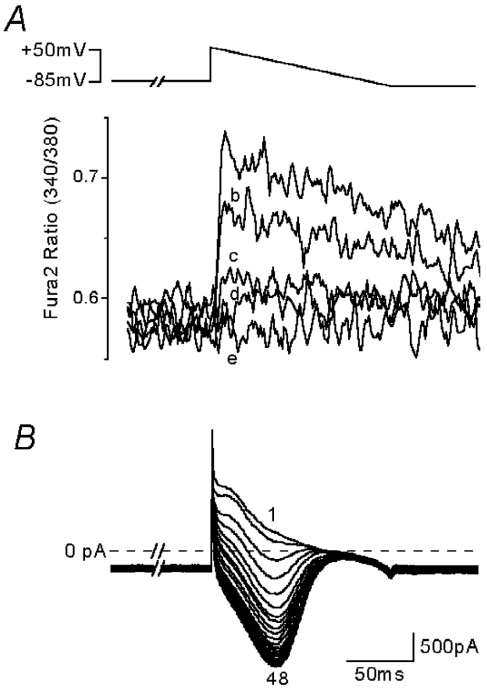

Fig. 1. Influence of heavy cytosolic Ca^{2+}-buffering with BAPTA on the $Ca^{2+}{}_i$ transient and membrane current. Isolated single rat ventricular myocytes were dialyzed with BAPTA (from 0 mM to 10 mM) added to the pipette and stimulated every 10 s with a 135 ms-ramp pulse from +50 mV to a holding potential of -85 mV after a patch-clamp in whole-cell configuration. In the pipette, KCl was replaced with 105 mM CsCl plus 20 mM TEA and 0.1 mM cAMP was also added. *A*. BAPTA concentration-related changes in global $Ca^{2+}{}_i$ transient. *B*. Representative actual current changes after dialysis with 10 mM BAPTA. 1: episode number 1, 48: episode number 48, a: no BAPTA, b: 1 mM BAPTA, c: 3 mM BAPTA, d: 5 mM BAPTA, e: 10 mM BAPTA.

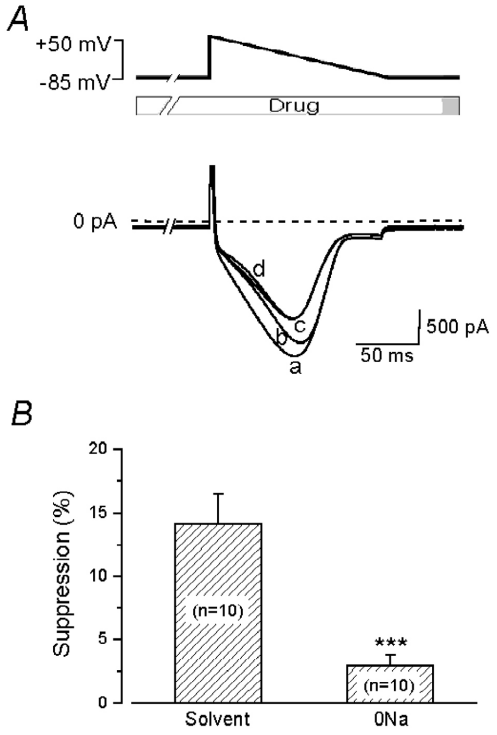

Fig. 2. Identification of the ryanodine-sensitive inward I_{NCX}. (A) Representative superimposed actual current changes. (B) Suppression of the ryanodine-dependent inward current after Na^+ depletion (0Na). Drugs were applied for 10 s until the end of test pulse. a: vehicle only, b: 10μM ryanodine, c: 0Na, d: 0Na plus 10 μM ryanodine. Parentheses are numbers of data. *** $p<0.001$, paired t-test with solvent only. Other legends are same as in Fig. 1.

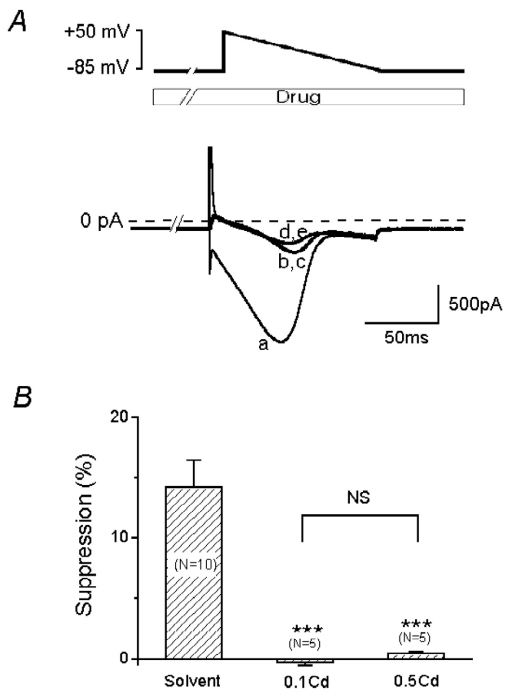

Fig. 3. Blocking of the ryanodine-dependent inward I_{NCX} by pretreatment with 0.1 mM and 0.5 mM $CdCl_2$. (A) Representative superimposed actual current changes. (B) Blocking of the ryanodine-dependent inward I_{NCX} by pretreatment with 0.1 mM and 0.5 mM $CdCl_2$. Ryanodine with no pretreatment came from Fig. 2. a: vehicle only, b: 0.1 mM $CdCl_2$, c: 0.1 mM $CdCl_2$ plus 10μM ryanodine, d: 0.5 mM $CdCl_2$, e: 0.5 mM $CdCl_2$ plus 10μM ryanodine. No pre: 10μM ryanodine only, 0.1 Cd: 0.1 mM $CdCl_2$ plus 10μM ryanodine, 0.5 Cd: 0.5 mM $CdCl_2$ plus 10μM ryanodine. Parentheses are numbers of data. *** $p<0.001$, unpaired t-test with solvent only. Other legends are same as in Fig. 1.

This control inward current was suppressed by 0.150± 0.024 pC/pF (14.2±2.3% of the control inward current) after 10μM ryanodine application suggesting that 14% of the inward current is dependent upon the Ca^{2+} release from SR. The involvement of forward-mode NCX was examined by using 0Na (Tyrode solution with Na^+ omission, see 'Methods') in this ryanodine-sensitive inward current. In the case of the NCX, high concentration of $NiCl_2$ or 0Na-0Ca (Tyrode solution with Na^+ and Ca^{2+} omission) is frequently used for suppression, as no specific blocker is yet available. However, only 0Na was used to suppress the forward-mode NCX in this case, because we did not want to block the CICR by concomitant omission of Ca^{2+}. The result showed that 10μM ryanodine-induced suppression of the inward current was strongly reduced to only 0.033±0.009 pC/pF (3.0±0.8% of the control inward current, $p<0.001$, Fig. 2B) after pretreatment with 0Na, suggesting that this ryanodine-sensitive inward current was actually an inward I_{NCX}. Therefore, these results suggest that the Ca^{2+} released from SR through the RyR caused 14% of the total inward current by being extruded from cell through forward-mode NCX after heavy cytosolic Ca^{2+}-buffering with 10 mM BAPTA in the rat ventricular myocytes.

To examine the involvement of the CICR in this ryanodine-sensitive inward I_{NCX} (RSII), I_{CaL} was blocked by a $CdCl_2$ application. In the case of $CdCl_2$, there is still a debate about its concentration required to completely block the I_{CaL}. Previous reports suggested 0.1 mM to be enough to completely block the I_{CaL} (Hobai et al, 1997a; Shen et al, 2000). On the other hand, recent reports have claimed that the I_{CaL} is not completely blocked by even 0.5 mM $CdCl_2$ or 60μM nifedipine (Griffiths & MacLeod, 2003; Trafford & Eisner, 2003). These results lead them to conclude that the VSRM is actually the CICR caused by this I_{CaL} remnant. Therefore, 0.1 mM and 0.5 mM were examined on this RSII in 5 cells. As shown in Fig. 3A, the control inward current was suppressed by 73.4±0.6% (0.864± 0.057 pC/pF) and 81.7±1.0% (0.961±0.063 pC/pF) after 0.1 mM and 0.5 mM $CdCl_2$ applications, respectively. After addition of 10μM ryanodine, the remaining inward current after $CdCl_2$ application was rather increased by 0.3±0.2% of the control inward current (0.005±0.003 pC/pF) in the case of 0.1 mM $CdCl_2$, and, it was slightly decreased by 0.4±0.1% of the control inward current (0.005±0.002 pC/pF) in the case of 0.5 mM $CdCl_2$. This result may indicate that the RSII was completely blocked by pretreatment with both concentrations of $CdCl_2$, as depicted in Fig. 3B. Therefore, it may be concluded from these results that, in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA, 0.1 mM $CdCl_2$ was enough to suppress the RSII, which was caused by the I_{CaL}-induced SR Ca^{2+} release. This conclusion is in line with the previous results

to suggest complete blocking of the I_{CaL} by 0.1 mM $CdCl_2$ (Hobai et al, 1997a; Shen et al, 2000).

Next, the possibility of an additional inward I_{NCX} was further examined by using 5 mM $NiCl_2$, a blocker of NCX and I_{CaL} (Hobai et al, 2000), from the 20~30% of the inward current remaining after a combined application of $CdCl_2$ and ryanodine in 5 cells. As shown in Fig. 4A, it was of interest to observe that 5 mM $NiCl_2$ further suppressed the remaining inward currents obtained after 0.1 mM and 0.5 mM $CdCl_2$ applications by 0.156±0.022 pC/pF (14.8±1.6% of the control inward current) and 0.054±0.013 pC/pF (5.1 ±1.2% of the control inward current), respectively. Therefore, this result may suggest the possibility of an additional inward I_{NCX}, constituting 15% of the control inward current, in addition to the RSII constituting 14% in the rat ventricular myocyte heavily Ca^{2+}-buffered with 10 mM BAPTA. Fig. 4B shows the I-V relationship of these 5 mM $NiCl_2$-sensitive inward I_{NCX}s calculated by subtracting the currents after 5 mM $NiCl_2$ combined with either 0.1 mM or 0.5 mM $CdCl_2$ from the remaining inward currents obtained after either 0.1 mM or 0.5 mM $CdCl_2$ application. Both the 5 mM $NiCl_2$-sensitive inward I_{NCX}s started from -50 mV until +40 mV with the peaks at -20 mV eliciting bell-shapes, but the height was higher in the case of 0.1 mM $CdCl_2$ pretreatment. Above +40 mV, the exact current was not measurable because of the capacitative current. This I-V relationship illustrates that the $NiCl_2$-sensitive inward I_{NCX} is apparently different from the I_{CaL}. Therefore, this I-V relationship also imply the presence of another inward I_{NCX} in addition to the RSII during the test pulse in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA.

The SR Ca^{2+} dependence was examined in this additional inward I_{NCX} identified by 5 mM $NiCl_2$ by using tetracaine, another SR Ca^{2+} release channel blocker but originally used as a local anestheitics (Overend et al, 1998; Mason & Ferrier, 1999). In this experiment, however, only 0.1 mM $CdCl_2$ was used, because, as shown in Fig. 3A and B, this concentration was enough for the suppression of the RSII, and its I-V relationship was similar regardless of the $CdCl_2$ concentration as shown Fig. 4B. Fig. 5A shows that the tetracaine further suppressed the inward current remaining after 0.1 mM $CdCl_2$ pretreatment in a dose-dependent manner (0.01~1 mM) by up to 0.163±0.019 pC/pF (15.6± 0.5% of the control inward current, n=9), a similar magnitude obtained after 5 mM $NiCl_2$ previously shown in Fig. 4A. However, both lidocaine and procaine, another local anesthetics, elicited no prominent suppression in the same dose ranges (Fig. 5B). These results suggest a possibility that the additional inward I_{NCX} identified by 5 mM $NiCl_2$ is also dependent on SR Ca^{2+} release.

The SR Ca^{2+} dependence of the additional inward I_{NCX} identified by 5 mM $NiCl_2$ was further examined after depletion of the SR Ca^{2+} storage by using 10 mM caffeine in 8 cells. As shown in Fig. 6A and B, 10 mM caffeine combined with 0.1 mM $CdCl_2$ suppressed the remaining inward current after 0.1 mM $CdCl_2$ to make it reach the current obtained after combined application of 1 mM tetracaine with 0.1 mM $CdCl_2$. Therefore, these results illustrated that the additional inward I_{NCX} identified by 5 mM $NiCl_2$ was also caused by the SR Ca^{2+} release in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA. Furthermore, the SR Ca^{2+} release in this case was probably mediated by a mechanism other than the CICR, as it was resistant to 10μM ryanodine.

The possible involvement of a mechanism other than the CICR was further examined in the tetracaine-sensitive inward I_{NCX} (TSII) by replacing the Ca^{2+} with Ba^{2+}, as Ba^{2+} does not induce the CICR (Nagasaki & Kasai, 1984; Lee, 1993; Adachi-Akahane et al, 1996). After replacing the $CaCl_2$ with equimolar $BaCl_2$, the equilibrated inward current was increased transmitting total charge influx of 0.773 ±0.096 pC/pF with a maximal current of 15.1±1.7 pA/pF (n=7) during stimulation with the test pulse. The equilibrated inward current was suppressed by 0.542±0.086 pC/pF (68.4±3.0%) and 0.570±0.086 pC/pF (72.2±2.5%) by applications with 0.1 mM and 0.5 mM $CdCl_2$, respectively. Still in this situation, however, 5 mM $NiCl_2$ combined with 0.1 mM or 0.5 mM $CdCl_2$ further suppressed the remaining inward current by 0.044±0.010 pC/pF (6.1± 1.5% of the control inward current) and 0.016±0.005 pC/pF (2.3±0.8% of the control inward current), respectively (Fig. 7A). The I-V relationship of this $NiCl_2$-sensitive inward I_{NCX}s also elicited patterns fairly similar to those obtained with normal $CaCl_2$ shown in Fig. 4B, although the inward current in the case with 0.5 mM $CdCl_2$ started and became maximal at slightly higher voltage than that with 0.1 mM $CdCl_2$ (Fig. 7C). Furthermore, Fig. 7B illustrates that this $NiCl_2$-sensitive inward I_{NCX} was resistant to 10μM ryanodine but sensitive to 1 mM tetracaine as like that obtained with normal $CaCl_2$. Therefore, the results obtained after replacement of $CaCl_2$ with $BaCl_2$ suggest that a mechanism

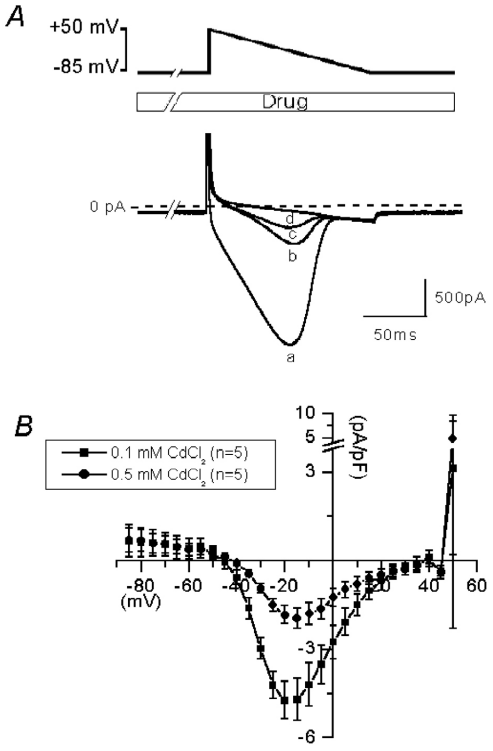

Fig. 4. Identification of an inward I_{NCX} by 5 mM $NiCl_2$ after pretreatment with 0.1 mM and 0.5 mM $CdCl_2$. (A) Representative superimposed actual current changes. (B) I-V relationships of the additional inward I_{NCX}s obtained after pretreatment with 0.1 mM and 0.5 mM $CdCl_2$. a: vehicle only, b: 0.1 mM $CdCl_2$, c: 0.5 mM $CdCl_2$, d: 5 mM $NiCl_2$. Parentheses are numbers of data. Other legends are same as in Fig. 1.

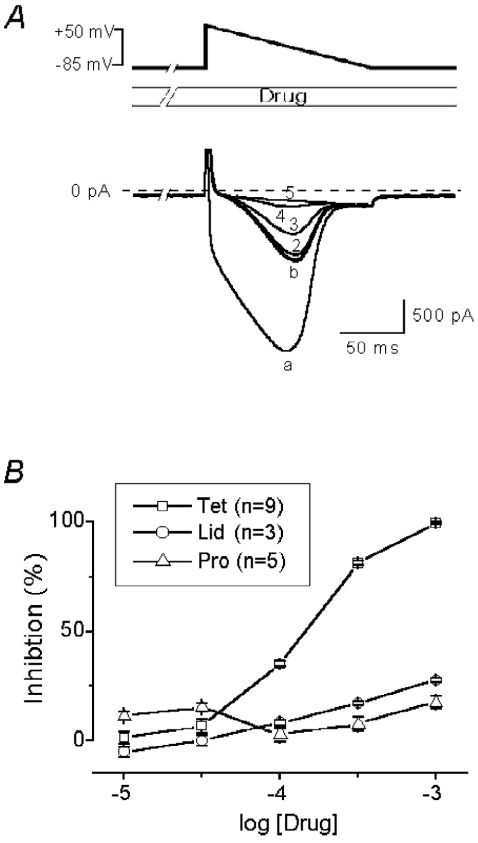

Fig. 5. Selective suppression of the remaining inward current obtained after 0.1 $CdCl_2$ by tetracaine among local anesthetics. (A) Representative superimposed actual current suppressions by tetracaine (0.01 mM～1 mM). (B) Concentration-related selectivity of tetracaine among local anesthetics. Data are expressed as % to the 5 mM $NiCl_2$-sensitive current in the presence of 0.1 mM $CdCl_2$. a: vehicle only, b: 0.1 mM $CdCl_2$, 2: 0.03 mM tetracaine plus 0.1 mM $CdCl_2$, 3: 0.1 mM tetracaine plus 0.1 mM $CdCl_2$, 4: 0.3 mM tetracaine plus 0.1 mM $CdCl_2$, 5: 1 mM tetracaine plus 0.1 mM $CdCl_2$. Tet: tetracaine, Lid: lidocaine, Pro: procaine. Parentheses are numbers of data. Other legends are same as in Fig. 1.

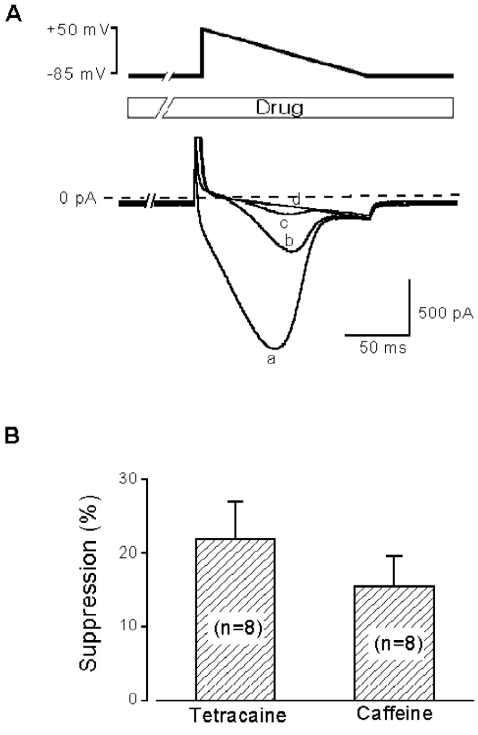

Fig. 6. Effect of SR Ca^{2+}-depletion with 10 mM caffeine on the 1 mM tetracaine-sensitive inward current in the presence of 0.1 mM $CdCl_2$. (A) Representative superimposed actual current changes, (B) Comparison of the magnitude of suppression between 1 mM tetracaine and 10 mM caffeine in the remaining inward current obtained after pretreatment with 0.1 mM $CdCl_2$. a: vehicle only, b: 0.1 mM $CdCl_2$, c: 10 mM caffeine plus 0.1 mM $CdCl_2$, d: 1 mM tetracaine plus 0.1 mM $CdCl_2$. Parentheses are numbers of data. Other legends are same as in Fig. 1.

other than the CICR is involved in the SR Ca^{2+} release in addition to the well-known CICR during stimulation with the test pulse in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA.

Fig. 8A shows the I-V relationships of the RSII obtained without $CdCl_2$ pretreatment and the TSII obtained with 0.1 mM $CdCl_2$ application. The RSII was calculated by subtracting the current obtained with 10μM ryanodine application from the control inward current (the current obtained with vehicle application) shown in Fig. 2B. The TSII was calculated by subtracting the current obtained with the application of 1 mM tetracaine in combination with 0.1 mM $CdCl_2$ from the current obtained with the application of only 0.1 mM $CdCl_2$ shown in Fig. 6A. The RSII was apparently inward from -20 mV over +40 mV with a peak at +15 mV eliciting a bell-shape. The TSII also elicited a bell-shape but it became inward starting from -50 mV to over +40 mV with a peak at-20 mV (i.e. 30～35 mV lower voltage than the RSII). The peak current was 2 times higher in the TSII than in the RSII (-6.2±0.5 pA/pF vs. -2.9±0.2 pA/pF). Above +40 mV, the exact currents were not measurable, because of the capacitative current in both cases. These profiles of I-V relationships clearly illustrate that these two inward I_{NCX}s are different currents. Therefore, these activation voltage profiles suggest that the RSII is equivalent to that of the I_{CaL}. On the other hand, the TSII identified in this study may be equivalent rather to that of the VSRM reported in the ventricular myocyte shortening, which started from -70～-65 mV with a peak at -20 mV (Ferrier & Howlett, 1995; Ferrier et al, 1998; Howlett et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Ferrier & Howlett, 2001).

Considering the present experimental condition, it is expected that the $[Ca^{2+}]_i$ in the case of the TSII would be determined solely by the Ca^{2+} released from the SR in the voltage under +40 mV, because of the following reason: The magnitude of the NCX is controlled by the driving force produced by two different determinants; (1) the potential difference between the reversal potential (E_{NCX}) and membrane potential (V_m), the E_{NCX}-V_m, and (2) the $[Na^+]$ and $[Ca^{2+}]$ gradients across the sarcolemma. In the present experimental situation, the former determinant (E_{NCX}-V_m) would decrease as the V_m increases from starting voltage to +40 mV, because the E_{NCX} is apparently higher than

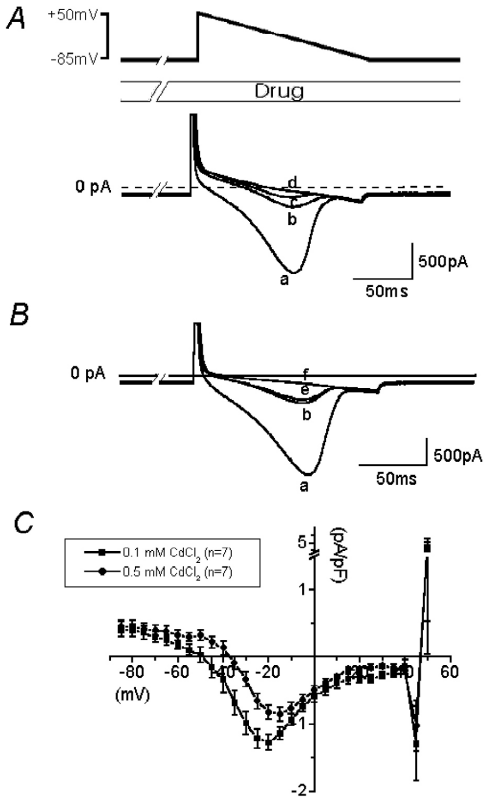

Fig. 7. Presence of the SR Ca^{2+}-dependent inward I_{NCX} obtained in the presence of $CdCl_2$ (0.1 mM and 0.5 mM) pretreatment even after replacement of $CaCl_2$ with $BaCl_2$. (A) Representative superimposed actual current changes, illustrating 5 mM $NiCl_2$-sensitive inward I_{NCX} after pretreatment with 0.1 mM and 0.5 mM $CdCl_2$, (B) Representative superimposed actual current changes, illustrating the tetracaine-sensitivity but ryanodine-insensitivity of the 5 mM $NiCl_2$-sensitive inward I_{NCX}, (C) I-V relationships of the 5 mM $NiCl_2$-sensitive inward I_{NCX}s obtained after pretreatment with 0.1 mM and 0.5 mM $CdCl_2$. a: vehicle only, b: 0.1 mM $CdCl_2$, c: 0.5 mM $CdCl_2$, d: 5 mM $NiCl_2$, e: 10μM ryanodine plus 0.1 mM $CdCl_2$, f: 1 mM tetracaine plus 0.1 mM $CdCl_2$. Parentheses are numbers of data. Other legends are same as in Fig. 1.

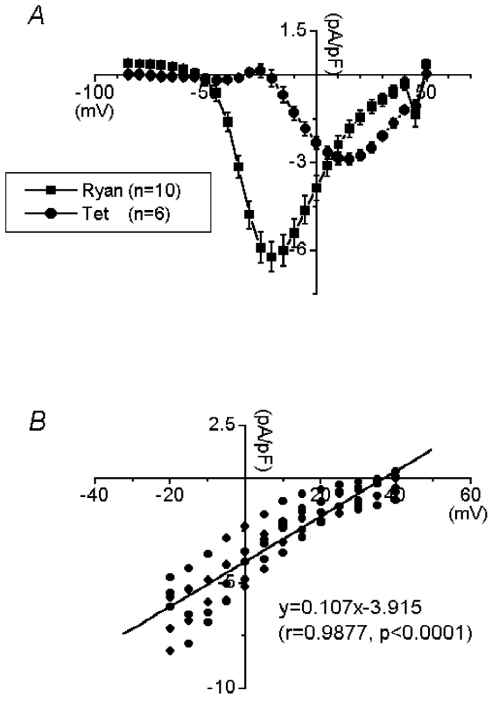

Fig. 8. I-V relationships of the ryanodine-sensitive inward I_{NCX} and the tetracaine-sensitive inward I_{NCX}. The ryanodine-sensitive inward I_{NCX} was calculated by subtracting the current after 10μM ryanodine application from the current after the vehicle application, shown in Fig. 2A. The tetracaine-sensitive inward I_{NCX} was calculated by subtracting the current after 1 mM tetracaine plus 0.1 mM $CdCl_2$ application from the current after 0.1 mM $CdCl_2$ application, shown in Fig. 5A. (A) I-V relationship. (B) Linear relationship of the tetracaine-sensitive inward I_{NCX} with V_m at voltages from -20 mV to +40 mV. Ryan: ryanodine-sensitive inward I_{NCX}, Tet: tetracaine-sensitive inward I_{NCX}. Parentheses are numbers of data.

+40 mV as shown in Fig. 8A. And, in the case of the latter determinant, the $[Ca^{2+}]_i$ becomes the only variable, because the $[Na^+]_o$ and $[Ca^{2+}]_o$ were already constant throughout the experiment (see 'Methods') and the $[Na^+]_i$ would also become constant as it returns to its baseline 10 ms after a stimulation in the heart (Matsuoka & Hilgemann, 1992). On the other hand, as mentioned in Fig. 3, since the I_{CaL} was effectively blocked by the $CdCl_2$ pretreatment, and the NCX was apparently inward in direction in the voltage under +40 mV, thus extruding Ca^{2+} from cell, the $[Ca^{2+}]_i$ would be determined solely by the amount of SR Ca^{2+} release. Therefore, it is expected that the decrease in the TSII after the peak (from -20 mV to +40 mV) would be attained by the V_m increase and/or the decrease of SR Ca^{2+} release.

To discern the exact cause of the TSII decrease between these two mechanisms, the linear relationship was examined between the TSII and V_m from the peak voltage of -20 mV to +40 mV using the least square method (Fig. 8B). The result shows that the inward I_{NCX} had a linear relationship with the V_m (y=0.107x-3.915, r=0.9877, $p < 0.0001$). This linear relationship suggests that the decrease in the inward current after the peak in the TSII was probably due to the increase of V_m rather than the decrease of the SR Ca^{2+} release. In other words, this conclusion may imply that the decrease of the SR Ca^{2+} release remains probably minimal constant after reaching the peak in the voltage range from -20 mV to +40 mV in the TSII. Therefore, this linear relationship indicates that the SR Ca^{2+} release increases to reach its peak, as the V_m increases from -50 mV to -20 mV, after which the SR Ca^{2+} release remains constant at the peak. Nevertheless, the SR Ca^{2+} release increases in a sigmoid shape as the membrane potential increases, although the current measured in this study elicited bell-shaped I-V relationship.

DISCUSSION

The major finding of this study is that the SR Ca^{2+}-dependent inward I_{NCX} was pharmacologically separable into two different fractions in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA. One was the RSII, which was an inward I_{NCX} sensitive to ryanodine and

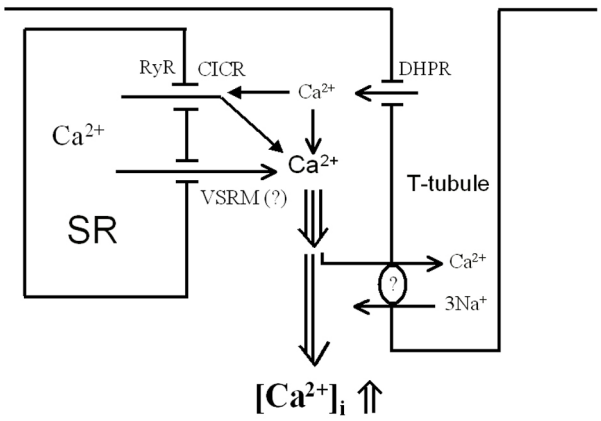

Fig. 9. Two different mechanisms suggested in the SR Ca^{2+} release in the heart. RyR: ryanodine receptor, CICR: Ca^{2+}-induced Ca^{2+} release, VSRM: voltage-sensitive release mechanism.

activated from -20 mV to over +40 mV with a peak at +15 mV in the absence of $CdCl_2$. The other was the TSII, which was an inward I_{NCX} insensitive to ryanodine but sensitive to tetracaine and activated from -50 mV to over +40 mV with a peak at -20 mV (in 30～35 mV lower voltages from start to the peak than the former) in the presence of 0.1 mM $CdCl_2$.

Micro-domanial interaction between NCX and Ca^{2+} from SR

The aim of this study was to directly monitor the SR Ca^{2+} release process in order to clarify the VSRM by using NCX as a Ca^{2+} sensor in the rat ventricular myocytes, because all the previous studies measured only ventricular myocyte shortening and/or global Ca^{2+}_i transient (Ferrier & Howlett, 1995; Ferrier et al, 1998; Howlett et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Piacentino et al, 2000; Ferrier & Howlett, 2001; Griffiths & MacLeod, 2003; Trafford & Eisner, 2003). To achieve this goal, the cytosolic Ca^{2+} of the isolated single rat ventricular myocytes was heavily buffered with 10 mM BAPTA after a patch-clamp in whole-cell configuration. 10 mM BAPTA was chosen, as it completely suppressed the global Ca^{2+}_i transient during the test pulse, as shown in Fig. 1A. It is expected in this situation that the Ca^{2+} diffusion time was limited short enough, thereby restricting any Ca^{2+} responses in a micro-domain separated from the global Ca^{2+}_i. Therefore, the SR Ca^{2+}-dependent inward I_{NCX} obtained during the test pulse in this study would reflect the SR Ca^{2+} release process, because the current represents an extrusion of Ca^{2+} from myocyte through the NCX immediately after its release from SR and before its diffusion to the global Ca^{2+}_i, as depicted in Fig. 9.

Heterogeneity in SR Ca^{2+} release process

If the SR Ca^{2+}-depndent inward I_{NCX} measured in this study truly reflects the SR Ca^{2+} release process, then the above mentioned major finding of this study could directly indicate that the SR Ca^{2+} release process is composed of two different processes. One is the ryanodine-sensitive process that could be obtained in the absence of $CdCl_2$ in the voltage range from -20 mV to over +40 mV with a peak at +15 mV, and the other is the ryanodine-insensitive but tetracaine-sensitive process that could be obtained in the presence of 0.1 mM $CdCl_2$ in the voltage range from -50 mV to over +40 mV with a peak at -20 mV (in 30～35 mV lower voltages from start to the peak than the former). The characteristics identified in this study indicate that the former is actually the SR Ca^{2+} release process mediated by the CICR, because the former, the RSII, was sensitive to ryanodine and to $CdCl_2$, suggesting that it is actually an I_{CaL}-dependent SR Ca^{2+} release. Its activation voltage range also supports this conclusion, because it was activated in the voltage range equivalent to the I_{CaL}.

However, the characteristics of the latter, the TSII, indicate that it is also an SR Ca^{2+} release-dependent inward I_{NCX}, but the SR Ca^{2+} release in this case does not seem to be triggered by the CICR, because of the following reasons: (1) The latter is also an inward I_{NCX}, because it was an inward current that was blocked by $NiCl_2$, a blocker of NCX and I_{CaL} (Hobai et al, 2000), after I_{CaL} suppression with either 0.1 or 0.5 mM $CdCl_2$ pretreatment. This result also suggests that the latter is not dependent upon the external Ca^{2+} influx through the L-type Ca^{2+} channel (I_{CaL}), because it was still present after I_{CaL} suppression with either 0.1 or 0.5 mM $CdCl_2$ pretreatment; (2) The activation voltage profile shown in Fig. 6A also supports the I_{CaL}-independence of the latter, because it started from -50 mV and became maximal at -20 mV, which was 30～35 mV lower voltages than the I_{CaL}; (3) The latter process was not sensitive to the ryanodine. Therefore, both 2 & 3 reasons indicate that the latter is not mediated by the CICR; (4) The ryanodine-insensitivity may imply that the latter has no relation with the SR Ca^{2+} release. Nonetheless, the results in this study illustrate that the latter was still SR Ca^{2+}-dependent, because it was suppressed by the SR Ca^{2+} storage depletion induced by 10 mM caffeine. The complete suppression of the latter by tetracaine, another SR Ca^{2+} release blocker, also supports the SR Ca^{2+}-dependence of the latter; (5) Lastly, the result obtained after replacement of Ca^{2+} with Ba^{2+}, which does not induce the CICR, illustrates the external Ca^{2+}-independence: As shown in Fig. 7A and C, the latter was still present, although Ca^{2+} was replaced with Ba^{2+}. Therefore, this result may imply that external Ca^{2+} influx is not required in the generation of the latter. This conclusion can also be extended to the Ca^{2+} entered the cell through the reverse-mode NCX. Based on all these pharmacological profiles, it is concluded that the latter is also a SR Ca^{2+} release process, but it is not dependent upon the external Ca^{2+} influx. Furthermore, the external Ca^{2+} influx-independence implies that the latter is mediated differently from the former SR Ca^{2+} release process by a mechanism other than the CICR.

Competence of the Tetracaine-sensitive inward I_{NCX} with the VSRM

Except the I-V relationship, all the characteristics of the latter, the TSII, obtained in this study, such as the external Ca^{2+} influx-independence, the resistance to high concentration (10μM) of ryanodine, and the selective sensitivity to tetracaine comply with the characteristics of the VSRM (Ferrier & Howlett, 1995; Hobai et al, 1997b; Ferrier et al, 1998; Mason & Ferrier, 1999; Ferrier et al, 2000; Ferrier & Howlett, 2001; Xiong et al, 2001). In the case of the I-V relationship, the TSII obtained in this study elicited a

bell-shape, while the VSRM reported in the ventricular myocyte shortening elicited a sigmoid voltage-contraction relationship. However, the linear relationship of the current decrease after the peak in TSII with the V_m, shown in Fig. 8B, revealed that the amount of Ca^{2+} release from SR has a sigmoid relation with voltage although the current elicited bell-shape in this study. Therefore, as depicted in Fig. 9, it may finally be concluded that the latter, the tetracaine-sensitive inward I_{NCX}, actually reflects the SR Ca^{2+} release process mediated by the voltage-sensitive release mechanism in the rat ventricular myocyte heavily Ca^{2+}-buffered with 10 mM BAPTA.

Discrepancies with the earlier studies

The involvement of I_{CaL} remnant: It has been reported that 0.1 mM $CdCl_2$ completely suppresses the I_{CaL} in the heart (Hobai et al, 1997a; Shen et al, 2000) and 0.1~0.5 mM $CdCl_2$ has frequently been used for suppression of the I_{CaL}. However, recent reports claimed that I_{CaL} suppression is not complete even with 0.5 mM $CdCl_2$ or 60μM nifedipine in the heart (Griffiths & MacLeod, 2003; Trafford & Eisner, 2003). Based on these results, they came to a conclusion that the VSRM shown after blocking of the I_{CaL} is actually the CICR induced by the I_{CaL} remnant. Furthermore, it has also been reported that this possibility would be more prominent in the presence of cAMP which is usually used in the experiments on the VSRM (Piacentino et al, 2000). If their claim were true, then pretreatment with 0.5 mM $CdCl_2$ should block the RSII, the CICR- mediated SR Ca^{2+} release process, in a magnitude stronger than that with 0.1 mM $CdCl_2$, because the magnitude of SR Ca^{2+} release is proportional to the amount of external Ca^{2+} influx (Fan & Palade, 1999). However, the present study, 0.1 mM $CdCl_2$ completely blocked the RSII, and no further suppression was noticed even after the $CdCl_2$ concentration was increased to 0.5 mM (Fig. 3A). Furthermore, the TSII was still present not only even after the suppression of RyR with 10μM ryanodine that was applied in combination with 0.1 $CdCl_2$ (Fig. 3A and Fig. 4A), but also after replacement of Ca^{2+} with Ba^{2+} that does not induce the CICR (Nagasaki & Kasai, 1984; Lee, 1993; Adachi-Akahane et al, 1996) shown in Fig. 5A&B. Therefore, the present results indicate that even 0.1 mM $CdCl_2$ is enough to suppress the I_{CaL} in the heavily Ca^{2+}-buffered rat ventricular myocyte (Hobai et al, 1997a; Shen et al, 2000). Further suppression of the control inward current after 0.5 mM $CdCl_2$ than that after 0.1 mM $CdCl_2$ might be due to the I_{NCX} suppression, because $CdCl_2$ also suppresses the I_{NCX} by 20%, 50%, and 60% at 0.1 mM, 0.3 mM, and 0.5 mM, respectively, in addition to its I_{CaL} suppression (Hobai et al, 1997a; Shen et al, 2000). Therefore, it may be concluded that the latter process is mediated by a mechanism other than the I_{CaL} remnant I_{CaL} remnant-mediated CICR in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mMB BAPTA.

Other discrepancies in experimental condition: It has been known that the VSRM-mediated contraction is not activated by cAMP itself, and requires a non-hydrolysable cAMP (Hobai et al, 1997b; Ferrier et al, 1998; Zhu & Ferrier, 2000). Only one report has shown that the VSRM-mediated shortening in the guinea-pig ventricular myocyte was selectively enhanced by amrinone, which increases the cAMP level by inhibiting phosphodiesterase III (Xiong et al, 2001). However, in that report, other cAMP raising drugs such as forskolin and IBMX did not show any selectivity on the VSRM. Nevertheless, in the present study, cAMP itself activated the VSRM-mediated SR Ca^{2+} release in a higher sensitivity than that mediated by the CICR.

Another discrepancy raised by this study is temperature. It has been suggested that activation of the VSRM-mediated contraction requires a physiological temperature of 37°C (Flesch et al, 1996; Ferrier & Howlett, 2001). However, this study was performed at room temperature (25°C), and had the VSRM-mediated SR Ca^{2+} release readily activated. The exact reason for these discrepancies cannot be found, as it was not further pursued in this study. Nevertheless, one possible reason might have been different measuring targets, since this study measured directly the SR Ca^{2+} release mediated by the VSRM, while previous studies measured the VSRM indirectly from ventricular myocyte shortening, in which several other mechanisms are involved after the SR Ca^{2+} release. These discrepancies remain to be cleared.

Implications in myocardial physiology

This study could differentiate the VSRM-mediated SR Ca^{2+} releases in the heavily Ca^{2+}-bufered rat ventricular myocytes in addition to that mediated by the CICR, and also showed that the magnitude of Ca^{2+} release from SR mediated by the VSRM was actually not smaller than that mediated by the CICR. Therefore, these findings provide not only a crucial evidence for the presence of the VSRM in the heart, but also a significant potential of the VSRM in the myocardial contraction. Furthermore, the conclusion derived from this study is significantly worthy for future evaluations of physiological and pathophysiological roles of the VSRM in myocardial contraction.

In conclusion, in the rat ventricular myocytes heavily Ca^{2+}-buffered with 10 mM BAPTA after a patch-clamp in whole-cell configuration, the SR Ca^{2+} release process monitored by the forward-mode Na^+-Ca^{2+} exchange is mediated by the voltage-sensitive release mechanism in addition to the Ca^{2+}-induced-Ca^{2+} release.

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